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ABSTRACT

Objectives: The physiological role of the contact system remains inconclusive. No obvious clinical complications have been observed for factor XII (FXII), prekallikrein (PK), or high molecular weight kininogen deficiencies even though the contact system *in vitro* is associated with coagulation, fibrinolysis, and inflammation. A global generation assay measuring the initial phase of the contact system could be a valuable tool for studies of its physiological role.

Design and methods: We investigated whether such a method could be developed using the principle of the Calibrated Automated Thrombin generation method as a template.

Results: A suitable kallikrein specific fluorogenic substrate was identified ($K_M = 0.91$ mM, $k_{cat} = 19$ s^{-1}), and kallikrein generation could be measured in undiluted plasma when silica was added as activator. Disturbing effects, including substrate depletion and the inner-filter effect, however, affected the signal. These problems were corrected for by external calibration with α_2-macroglobulin-kallikrein complexes. Selectivity studies of the substrate, experiments with FXII and PK depleted plasmas, and plasma with high or low complement C1-esterase inhibitor activity indicated that the obtained and calibrated signal predominantly was related to FXII-dependent kallikrein activity.

Conclusions: The findings described show that establishment of a kallikrein generation method is possible. Potentially, this setup could be used for clinical studies of the contact system.

1. Introduction

Four plasma proteins comprise the contact activation system [1]. In plasma, prekallikrein (PK) and factor XI (FXI) are mostly found in complex with the non-enzymatic co-factor high molecular weight kininogen (HMWK) [2,3]. Factor XII (FXII), on the other hand, circulates unbound. The contact system is initiated when encountered with activating surfaces. While the exact mechanism is unknown, the surface causes a conformational change in FXII rendering it susceptible for cleavage [4]. Once cleaved, activated FXII (FXIIa) can activate neighboring PK and FXI bound to the surface through HMWK. Amplification of the system follows from the ability of kallikrein to generate FXIIa [5]. Several protease inhibitors regulate the contact system. Complement C1-esterase inhibitor (C1-inhibitor) is predominating [6], but contributions from α_2-macroglobulin (α_2M) and other inhibitors have been observed [7,8]. *In vitro* experiments reveal connections between the contact system and coagulation, fibrinolysis, and inflammation. FXI serves as the link between contact activation and coagulation as its activation initiates the intrinsic pathway of coagulation and the formation of thrombin [9]. *In vivo*, FXII, PK, and HMWK deficiencies are not associated with bleeding tendencies, questioning the role of the contact system in normal haemostasis [1]. Recent studies, however, have shown that FXII deficient mice are protected from occlusive thrombi in experimental thrombosis models [10]. Other studies suggest inhibition of FXII and FXI as alternative anti-coagulant approaches [11]. Decreased or absent C1-inhibitor activity is associated with symptoms that are mainly related to activation of inflammation [1].

A sensitive global activity assay measuring contact activation is currently not available, and in general, there is only a limited assortment of biomarker assays available to measure the contact system [12–17]. Studies of the connection between the contact system and clinical

conditions are therefore often hampered. A global generation test that measures the contact system may, potentially, facilitate such investigations.

The Calibrated Automated Thrombin generation method (CAT-method) is a global generation test that utilizes a fluorogenic substrate to measure the thrombin generation potential in human plasma after initiation of the extrinsic pathway of coagulation [18]. The CAT-method has proven to be a valuable tool to study disturbances of the coagulation system in vitro, and it shows potential for routine clinical application [19]. The use of a synthetic fluorogenic substrate to measure thrombin generation in undiluted plasma is, however, associated with several complications such as substrate depletion, the inner-filter effect, and cleavage of substrate by α_2M-thrombin [20]. In the CAT-method, these complications have been circumvented by unique approaches developed by Hemker and colleagues [18,21]. We investigated if the CAT-method could be used as a template for a method to measure contact activation through kallikrein generation after initiation of the contact system.

2. Materials and methods

2.1. Buffers

"BSA60 buffer" (20 mM Hepes, 0.02% NaN$_3$, 60 g/L bovine serum albumin (BSA), pH = 7.4). "BSA5 buffer" (20 mM Hepes, 0.02% NaN$_3$, 5 g/L BSA, pH = 7.4). "BSA-blank buffer" (20 mM Hepes, 150 mM NaCl, 0.02% NaN$_3$, pH = 7.4). "Gel filtration buffer" (20 mM Hepes, 300 mM NaCl, 0.02% NaN3, pH = 7.4). "SDS sample buffer" (2× Laemmli Sample Buffer (Bio-Rad, Hercules, California, U.S.)). "PBS-TW buffer" (Phosphate-buffered-saline (PBS), 0.05% Tween-20, pH = 7.4).

2.2. Plasma

Sodium citrate (0.109 M) and ethylenediaminetetraacetic acid (EDTA) stabilized plasma pools were obtained from 12 healthy individuals, who did not take hormone supplements. Plasma was frozen at −80 °C in 300 μL ampoules. FXII depleted plasma (FXII DEP) was obtained from Haematologic Technologies Inc. (HTI, Essex Junction, Vermont, USA). Prekallikrein depleted plasma (PK DEP) was obtained from Affinity Biologicals (Ancaster, Ontario, Canada). EDTA stabilized plasma was obtained from two hereditary angioedema (HAE) patients.

2.3. Reagents and materials

Corn trypsin inhibitor (CTI), 2 mg/mL, was obtained from HTI. Soybean trypsin inhibitor (SBTI) was obtained from Sigma-Aldrich Denmark ApS (Sigma, Brøndby, Denmark). Twenty-five milligram of lyophilized SBTI was dissolved in 5 mL distilled water. Human complement C1-esterase inhibitor (C1-inhibitor) was obtained from CSL Behring (Lyngby, Denmark), dissolved in 10 mL H$_2$O, and dialyzed towards 5 L PBS at 4 °C for 72 h in a Spectra/Por® Dialysis Membrane molecular weight cut-off (MWCO) 6–8000 (Spectrumlabs®, Rancho Dominquez, Ca, U.S.).

Affinity purified kallikrein and FXIIa from human plasma were obtained from Enzyme Research Laboratories (Swansea, UK). One milligram of lyophilized protein was dissolved in 1 mL distilled water. Plasmin from human plasma was obtained from Sigma. One milligram was dissolved in 1 mL of distilled water containing 50% glycerol. Human FXIa, 2.0 mg/mL in 50% glycerol, and human α-thrombin, 7.8 mg/mL in 50% glycerol, were obtained from HTI. BSA was from Sigma.

Kallikrein sensitive chromogenic substrate, S-2302, was obtained from Chromogenix (Mölndal, Sweden). Twenty-five milligram of substrate was dissolved to a concentration of 4 mM in distilled water. Fluorogenic substrate, OmniCathepsin fluorogenic substrate

(Cbz-Phe-Arg-AMC), was obtained from Enzo Life Science (Exeter, UK). Fluorogenic substrate, Cbz-Pro-Phe-Arg-AMC, was synthesized on request by Chempeptide Limited (Shanghai, China). Fluorogenic substrate, Boc-Leu-Lys-Arg-AMC, was obtained from Bachem (Bubendorf, Switzerland). All fluorogenic substrates were dissolved in DMSO to a concentration of 50 mM (stock solutions).

PK-11 and PK-32: in-house produced mouse monoclonal antibodies against the heavy-chain of human prekallikrein/kallikrein. Nonsense antibody: mouse anti-human osteoclast-associated receptor (OSCAR) antibody [22].

APTT reagent, STA®-PTT Automate 5, was obtained from Triolab A/S (Brøndby, Denmark). The powder was dissolved in 5 mL BSA60 buffer (stock solution). Dextran sulfate sodium salt, with a molecular weight of 500 kDa, was obtained from Pharmacia AB (Uppsala, Sweden). Stock solution of dextran sulfate was prepared by dissolving 2 g of dextran sulfate in 1 L of distilled water. Colloidal silica LUDOX AS-40, a 40% (w/v) silica suspension in H$_2$O, was obtained from Sigma (stock solution). Polyethylene glycol with an average molecular weight of 6000 Da (PEG-6000) was obtained from Sigma.

Hiprep 16/60 Sephacryl S-200 HR column (Sephacryl column) was obtained from GE Healthcare Europe GmbH (GE Healthcare, Brøndby, Denmark). Vivaspin 20™, 100 kDa MWCO, sample concentrator (Vivaspin sample concentrator) was obtained from GE Healthcare. Immulon 2HB round-bottom 96-well plate (2HB plate) was obtained from Thermo Scientific (Rochester, NY, U.S.). Fluoroskan Ascent™ Microplate Fluorometer (fluorometer) was obtained from Thermo Scientific. Thrombinoscope™ software was obtained from Thrombinoscope BV (Maastricht, Netherlands).

2.4. Fluorogenic substrates

The kinetic constants were determined essentially as described elsewhere [23]. The fluorogenic substrate Boc-Leu-Lys-Arg-AMC was diluted from stock solution in BSA60 buffer to obtain a final concentration of 1000, 800, 600, 400, 200, 100, 50, or 0 μM in the well. For Cbz-Pro-Phe-Arg-AMC and Cbz-Phe-Arg-AMC, the final substrate concentration was 2000, 1800, 1400, 1000, 800, 600, 400, or 0 μM in the well. The enzyme to be investigated was diluted from stock solution in BSA5 buffer. The final concentrations were 5.8 nM (kallikrein), 125 nM (FXIIa), 60.25 nM (plasmin), 272.5 nM (thrombin), or 3.125 nM (FXIa) in the well. α_2M-kallikrein was diluted 1:4 in BSA5 and added to the plate (final dilution of 1:24 in the well). The kinetic parameters were determined using the following setup; 100 μL of fluorogenic substrate was added to a 2HB plate and the plate was warmed for 5 min, at 37 °C, in the fluorometer. Twenty μL of enzyme solution (preheated to 37 °C) was then added using the fluorometer dispenser just before measurements were started. Each substrate concentration was run in quadruple and hydrolysis was followed for 40 min. Michaelis-Menten curves were fitted to the obtained data. k_{cat} was calculated from the determined V_{max}, by first converting the fluorescence units per time to concentration per time, using a calibration curve, and then dividing this rate by the enzyme concentration.

2.5. Calibrator

2.5.1. Preparation of α_2M-kallikrein complexes

Human α_2M was obtained using a slight modification of a procedure described elsewhere [18,24]: To 1 vol of human plasma, 0.28 vol PEG-6000 (25% in H$_2$O) was added and the mixture was incubated for 30 min. After centrifugation (3000g), the precipitate was discarded and the supernatant was mixed with an additional 0.72 vol PEG-6000 (25% in H$_2$O). After 30 min incubation, the mixture was centrifuged (3000g) and the precipitate was dissolved in 0.1 vol BSA-blank buffer ("crude α_2M"). To prepare α_2M-kallikrein complexes, crude α_2M was diluted 1:8 in BSA-blank buffer and 100 μg/mL of kallikrein (final concentration) was added. The mixture was incubated overnight (ON)

Table 1
Investigation of kallikrein fluorogenic substrates.
The kinetic constants of human kallikrein towards three fluorogenic substrates.

Peptide sequence	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\,s^{-1}$)
Cbz-Phe-Arg-AMC	0.83 (0.68–1.1)	33 (31–37)	$4.0 \cdot 10^4$
Cbz-Pro-Phe-Arg-AMC	1.4 (0.99–1.5)	45 (39–47)	$3.2 \cdot 10^4$
Boc-Leu-Lys-Arg-AMC	0.91 (0.84–0.96)	19 (18–20)	$2.1 \cdot 10^4$

The results are presented as median and range of quadruple determinations performed in buffer with high albumin concentration.

at 4 °C. Formed complexes were then purified by gel filtration using a Sephacryl column equilibrated with gel filtration buffer. The fractions containing α_2M-kallikrein were pooled, concentrated, using Vivaspin sample concentrators, and diluted in BSA5 buffer. The kallikrein-like activity of the calibrator was adjusted to 200 nM, using chromogenic substrate S-2302, and C1-inhibitor (200 µg/mL, final concentration), SBTI (150 µg/mL, final concentration), and CTI (120 µg/mL, final concentration) were added to prevent kallikrein generation in the calibrator sample when used in the method.

2.5.2. Western blot analysis

Samples of kallikrein (100 µg/mL), crude α_2M (1:8), a mixture of crude α_2M (1:8) and kallikrein (100 µg/mL), as well as the pooled and concentrated fractions from gel filtration were diluted 1:10 in PBS-TW buffer and subsequently 1:2 in SDS sample buffer. All samples were heated for 8 min at 96 °C to denature proteins. The samples were separated by SDS-PAGE with a 4–15% MiniPROTEAN® TGX™ precast gel (Bio-Rad) using SDS running buffer (Bio-Rad), as described by the manufacturer, for 60 min at 150 V. The proteins were transferred to a polyvinylidene fluoride membrane with a Trans-Blot® Transfer Pack (Bio-Rad) using the Trans-Blot® Turbo™ Blotting System (Bio-Rad). The membrane was afterwards blocked for 30 min, with PBS-TW buffer, and incubated ON at 4 °C with PK-11 (diluted to a final concentration of 0.66 µg/mL in PBS-TW buffer). Next, the membrane was washed three times with PBS-TW buffer and incubated for 1 h with horseradish peroxidase conjugated rabbit anti-mouse IgG antibody (Zymax™, Invitrogen) diluted 1:4000 (v/v) in PBS-TW buffer. Proteins were visualized using 0.4 mg/mL of 3-amino-9-ethylcarbazole in 50 mM acetate buffer (pH = 5.0) with 0.015% H_2O_2.

2.5.3. Stability of α_2M-kallikrein complexes

200 µL of α_2M-kallikrein was added to 800 µL of citrate plasma pool. The mixture was placed in a water bath (37 °C). At times zero, 30 min, and 60 min, a sample of 100 µL was withdrawn and added to a 2HB plate. The plate was warmed for 5 min in the fluorometer. Immediately before measurements were initiated, Boc-Leu-Lys-Arg-AMC was added to pre-warmed BSA60 buffer and 20 µL of the solution was added to the wells. The final substrate concentration was 0.42 mM. The fluorescence development of the samples was recorded every 12 s, and the initial velocity of the first 3 min was calculated. For each time point, the initial velocity was determined as the mean of three repetitions.

2.6. Activating reagent

The activators under study were tested in the following setup: 20 µL of BSA5 together with 80 µL of citrate pool were added to a 2HB plate

and the plate was warmed in the fluorometer to 37 °C. A start reagent for each activator was prepared by diluting the stock solutions of the activators in BSA60 buffer; Dextran sulfate was diluted to 600 µg/mL and silica to 6 mg/mL. The APTT reagent was not diluted. Lower concentrations of the activators were prepared by further dilutions in BSA60. Finally, the fluorogenic substrate was added to each reagent at a concentration of 2.5 mM. Twenty microliter of the solutions were then added to the plate and fluorescence was measured every 12 s for 20 min. Each activator concentration was run in duplicate. The final concentrations of the activators in the well were; dilutions 1:6, 1:12, 1:18, and 1:24 for the APTT reagent, 100, 50, 25, and 12.5 µg/mL for dextran sulfate, and 1, 0.5, 0.25, 0.125, or 0 mg/mL for silica.

2.7. Calculations

The raw data obtained from a generation experiment in a citrate pool (obtained as described in Section 2.8) were transferred to a Microsoft Excel spreadsheet. The corrected calibrator curve was found from a 6th-degree polynomial fit of the recorded calibrator data. The relation between the corrected and measured calibrator curves was calculated [18] and used to convert the obtained fluorescence units of the generation sample into corrected fluorescence units. The contribution from α_2M-kallikrein was subtracted as described previously [25]. The fluorescence units of the corrected generation curve were converted to enzyme concentration by comparing them to the calibrator initial velocity. The calculations were also conducted using the Thrombinoscope software for comparison.

2.8. Validation of kallikrein generation

Calibrated kallikrein generation was determined using four wells for each plasma sample tested. Two wells were used to determine kallikrein generation (generation wells) and two wells were used for calibration of signal (calibrator wells). To generation wells, 20 µL of BSA5 buffer containing either 90 µg/mL of PK-32, 90 µg/mL of nonsense antibody, 480 µg/mL of C1-inhibitor, or buffer alone was added. To calibrator wells, 20 µL of α_2M-kallikrein, with an activity corresponding to 200 nM kallikrein, was added. Next, 80 µL of the plasma samples to be tested were added to the wells and the plate was warmed to 37 °C. The following plasmas were tested: Citrate pool, FXII DEP, PK DEP, HAE plasma, and mixtures of the citrate pool and FXII DEP consisting of 50%, 75%, and 90% FXII DEP. Immediately before initiation of experiment, colloidal silica and Boc-Leu-Lys-Arg-AMC were added to BSA60 buffer preheated to 37 °C. Twenty microliter of the solution was added to the wells. The final concentrations of silica and substrate were 0.24 mg/mL and 0.42 mM, respectively. Fluorescence was measured every 12 s for 25 min and calculations were conducted using the Thrombinoscope software.

3. Results

3.1. Fluorogenic substrate

The Michaelis-Menten kinetics of three fluorogenic substrates for kallikrein [26] were determined in BSA60 buffer and the results are listed in Table 1. Boc-Leu-Lys-Arg-AMC displayed lower k_{cat}

Table 2
Selectivity study of substrate Boc-Leu-Lys-Arg-AMC.
Kinetic constants of human kallikrein, FXIIa, FXIa, thrombin, and plasmin towards Boc-Leu-Lys-Arg-AMC are shown.

	Kallikrein	Factor XIIa	Factor XIa	Thrombin	Plasmin
K_M (mM)	0.91 (0.84–0.96)	0.92 (0.81–1.0)	3.4 (3.3–3.4)	0.74 (0.62–0.89)	3.8 (3.5–4.0)
k_{cat} (s^{-1})	19 (18–20)	0.03 (ND)	28 (28–29)	0.02 (ND)	3.7 (3.4–3.8)
k_{cat}/K_M ($M^{-1}\,s^{-1}$)	$2.1 \cdot 10^4$	33	$8.2 \cdot 10^3$	27	974

The results are presented as median and range of quadruple determinations performed in buffer with high albumin concentration. ND: not determined.
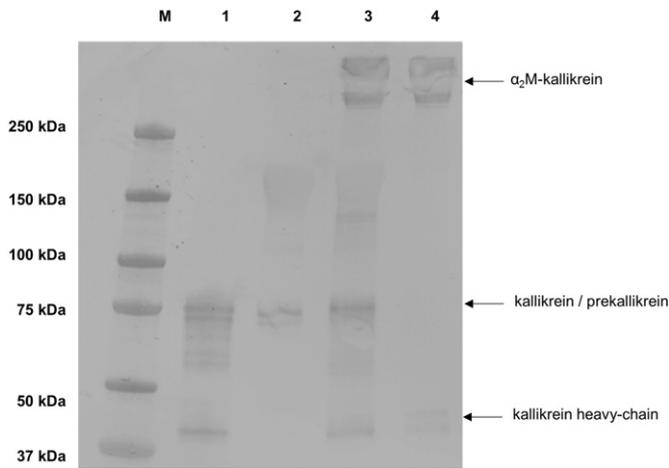
Fig. 1. Western blot analysis of α_2M-kallikrein complex (calibrator) preparation. The samples include M: Molecular marker (Precision Plus Protein™ Kaleidoscope™ Standards, Bio-Rad) 1: Kallikrein (5 μg/mL, final concentration), 2: "Crude α_2M" (1:160, final concentration), 3: "Crude α_2M" incubated with kallikrein (1:160, final concentration), and 4: Pooled fractions from gel filtration (1:160, final concentration). The western blot was visualized using PK-11.

Boc-Leu-Lys-Arg-AMC and Cbz-Phe-Arg-AMC showed similar binding affinities with apparent K_M values of 0.91 and 0.83 mM, respectively. With the aim of obtaining a substrate with a low k_{cat} and a high K_M [27], Boc-Leu-Lys-Arg-AMC was selected for further studies.

In BSA60 buffer, the kinetic constants of the substrate Boc-Leu-Lys-Arg-AMC for other contact system related serine proteases in plasma were investigated. In Table 2, these constants are compared with those obtained for kallikrein. For FXIa, a K_M of 3.4 mM and a k_{cat}/K_M of $8.2 \cdot 10^3 \, M^{-1} \, s^{-1}$ were observed. For FXIIa and α-thrombin, K_M values of 0.92 mM and 0.74 mM, respectively, were found with a k_{cat}/K_M for both enzymes of around $30 \, M^{-1} \, s^{-1}$. Plasmin showed K_M and k_{cat}/K_M values of 3.8 mM and $974 \, M^{-1} \, s^{-1}$, respectively.

3.2. Calibrator

A western blot (Fig. 1) demonstrated assembly of α_2M-kallikrein complexes. A kallikrein related high molecular weight band was observed in the lane containing a mixture of "crude α_2M" and kallikrein (lane 3). This band was not present in lanes including only kallikrein (lane 1) or "crude α_2M" (lane 2). After gel filtration, fractions containing only the kallikrein related high-molecular weight protein were obtained (lane 4).

In plasma, the initial amidolytic activity of α_2M-kallikrein towards the substrate, Boc-Leu-Lys-Arg-AMC, was constant for at least 1 h (data not shown). The data confirm that α_2M-kallikrein complexes

($19 \, s^{-1}$) for kallikrein compared to Cbz-Phe-Arg-AMC ($33 \, s^{-1}$) and Cbz-Pro-Phe-Arg-AMC ($45 \, s^{-1}$). Cbz-Pro-Phe-Arg-AMC displayed the lowest binding affinity with an apparent K_M value of 1.4 mM whereas

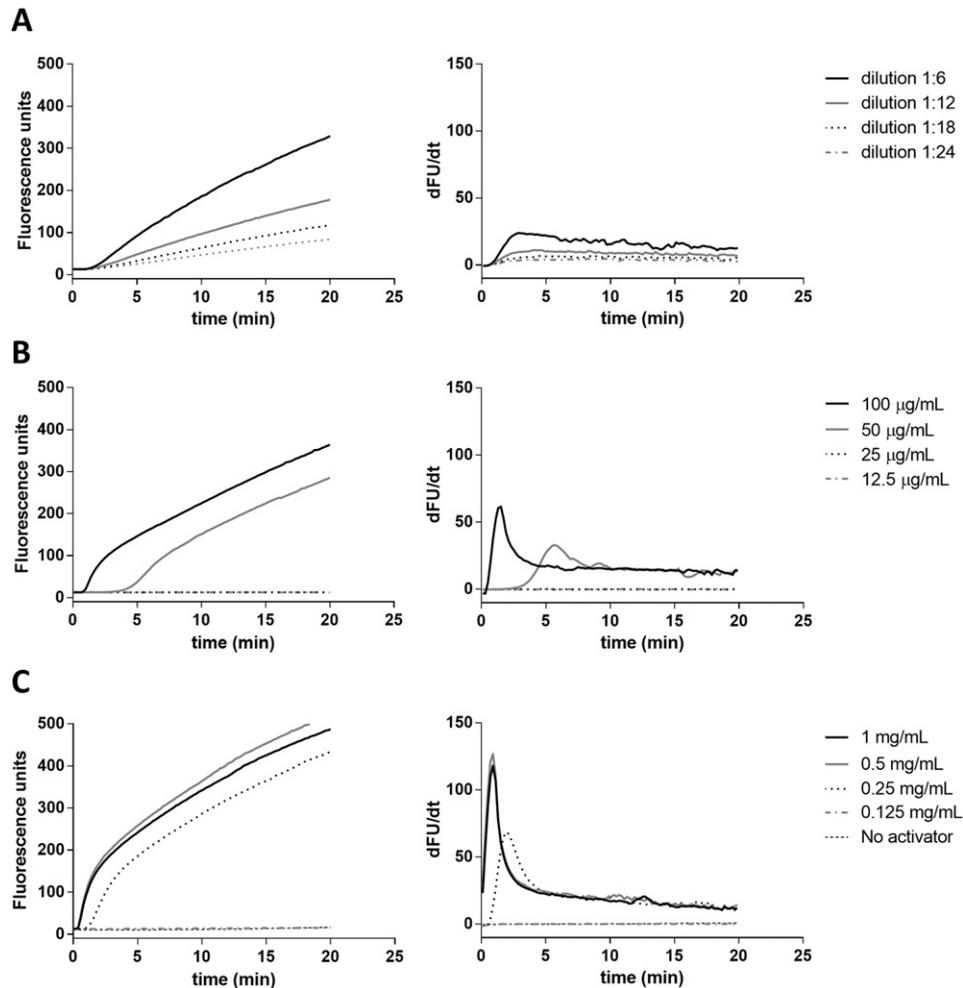**Fig. 2.** Generation of signal in a citrate pool by different activating reagents. A: APTT reagent, STA®-PTT Automate 5, at final dilution of 1:6, 1:12, 1:18, and 1:24. B: Dextran sulfate at final concentration of 100, 50, 25, and 12.5 μg/mL. C: Silica at final concentration of 1, 0.5, 0.25, 0.125 mg/mL, or no activator. Raw data (left) and first derivative (right) are shown. Each line represents the mean of duplicate measurements.

possess enzymatic activity towards small substrates that are protected from other inhibitors in plasma.

In BSA60 buffer, the apparent K_M of α_2M-kallikrein towards Boc-Leu-Lys-Arg-AMC was determined to 0.58 mM. This is somewhat lower than the apparent K_M of free kallikrein (0.91 mM).

3.3. Activating reagent

The potential of different activators to generate a measureable signal in the current setup is shown in Fig. 2. The results demonstrate a low activation potential of the APTT reagent (Fig. 2A). Both dextran sulfate (Fig. 2B) and colloidal silica (Fig. 2C) were potent activators. However, substantial turbidity of the sample followed the use of dextran sulfate indicating precipitation of proteins in the mixture (data not shown). This was not the case when silica was used as activator. No signal generation was observed in the absence of activator (Fig. 2C, dashed black line).

3.4. Calculations

The recorded fluoresence of the calibrator demonstrated a decreasing reaction velocity at fixed enzyme concentration (Fig. 3A, black line). The "correct" calibrator curve was determined (Fig. 3A, dotted line), and the relation between the corrected and the observed calibrator curve was found (Fig. 3B). Application of this relation to the raw generation data (Fig. 3C, dotted line) resulted in a steady end-level signal (Fig. 3C, black line). Measurements of substrate cleavage by α_2M-kallikrein, formed during the generation, were then substracted (Fig. 3D, black line). Finally, the fluorescence units were converted to enzyme concentration (Fig. 3E, grey dotted line). This curve was identical to the curve obtained when the calculations were performed by the Thrombinoscope software (Fig. 3E, black dotted line).

3.5. Validation of kallikrein generation

Fluorescence development was absent in plasmas depleted of FXII or PK while signal generation was recorded in a citrate pool (Fig. 4A). Addition of C1-inhibitor to a citrate pool diminished the fluorescence generated considerably compared to a citrate pool without added C1-inhibitor (Fig. 4B). PK-32 also diminished the generation signal whereas a very modest effect was seen when a nonsense antibody was added to the plasma (Fig. 4B). Furthermore, the signal obtained declined with decreasing levels of FXII (Fig. 4C). A substantially higher signal
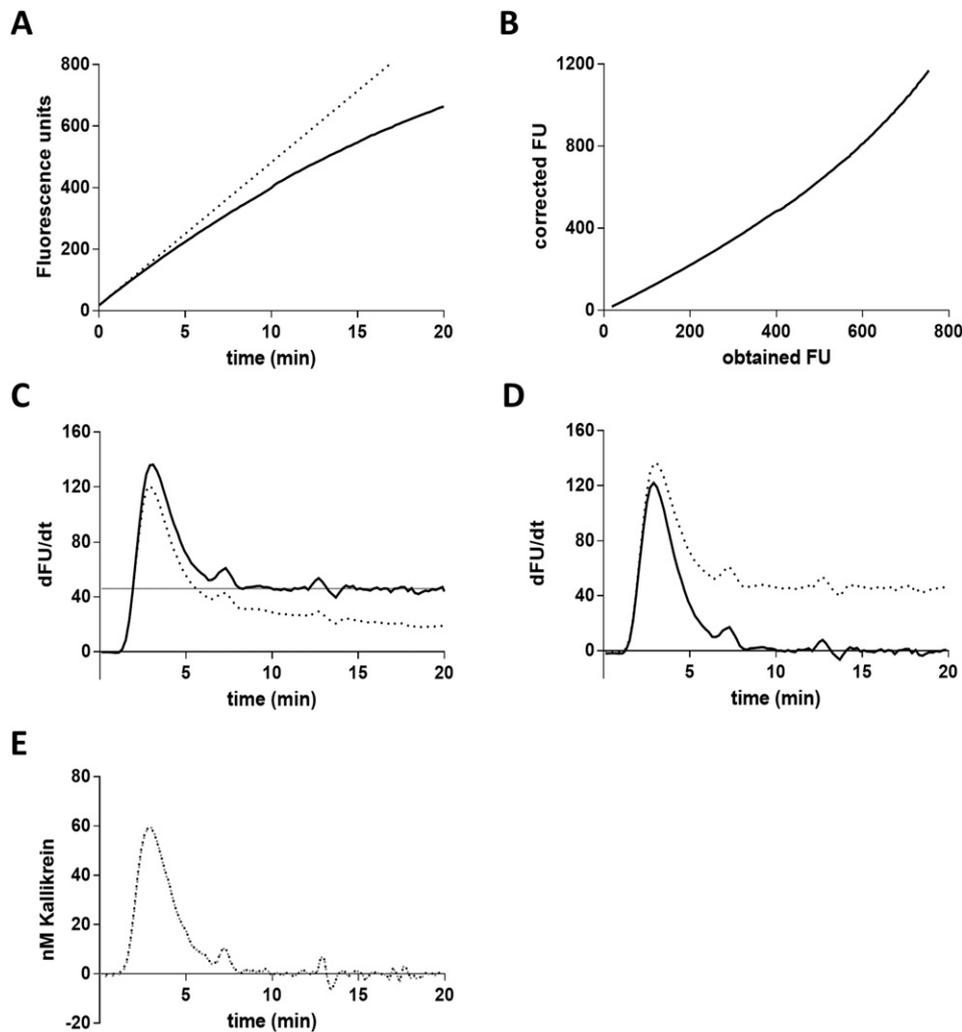

Fig. 3. Application of the mathematical algorithms to correct for the effects from substrate depletion, the inner-filter effects, and cleavage of substrate by α_2M-kallikrein. The raw data were obtained from a generation experiment in a citrate pool with silica as activator (0.24 mg/mL, final concentration), Boc-Leu-Lys-Arg-AMC (0.42 mM, final concentration) as substrate, and α_2M-kallikrein as calibrator (33.3 nM kallikrein-like activity, final concentration). A: The recorded fluorescence from the calibrator sample (black line) and the "corrected" calibrator curve (dotted line), which was calculated from a 6th-degree polynomial fit of the recorded fluorescence. B: Plot of the raw (measured) data versus the corrected calibrator data. C: First derivate of the raw data from the generation sample (dotted line) and the "corrected" generation data (black line), which was calculated using the relation observed in B. D: The corrected generation data before (dotted line) and after (black line) subtraction of α_2M-kallikrein contribution. E: The final curve as obtained by manual calculations (grey dotted line) and by the Thrombinoscope software (black dotted line).
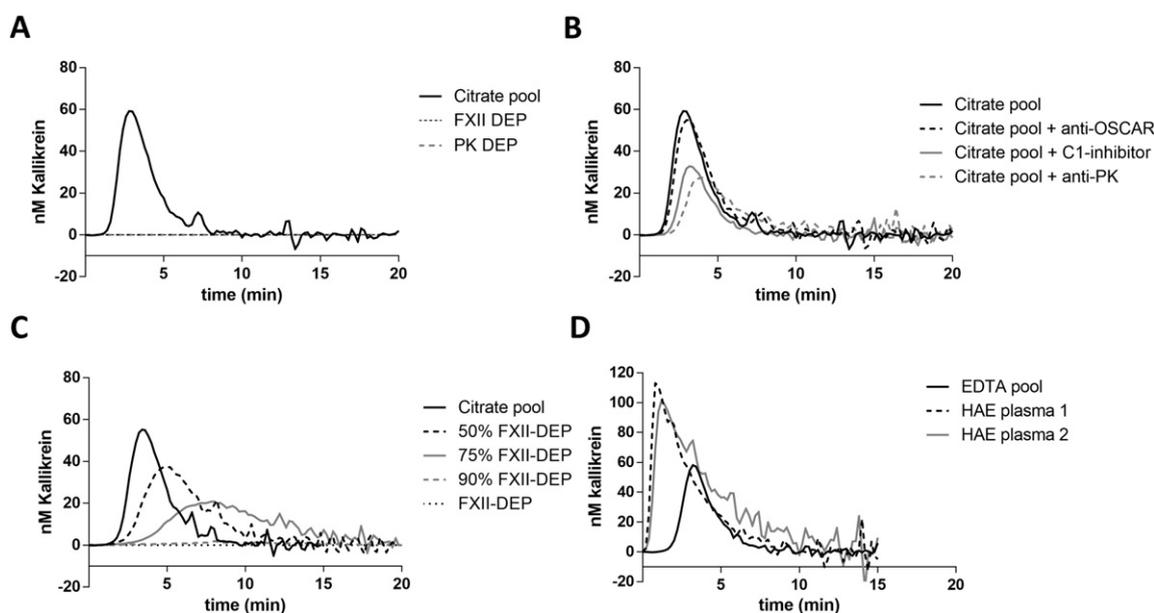
Fig. 4. Validation of kallikrein generation. A: Signal generation in FXII depleted plasma (FXII DEP), prekallikrein depleted plasma (PK DEP), and a citrate pool is shown. B: Signal generation in a citrate pool with addition of C1-inhibitor (80 μg/mL, final concentration), PK-32 (15 μg/mL, final concentration), nonsense antibody (15 μg/mL, final concentration), or buffer alone is presented. C: Signal generation in FXII-DEP, citrate pool as well as FXII-DEP mixed with 50%, 25%, 10%, and 0% citrate pool. D: Signal generation in EDTA pool and HAE plasmas is shown. All experiments were done using silica as activator (0.24 mg/mL, final concentration), α_2M-kallikrein as calibrator (33.3 nM kallikrein-like activity, final concentration), and Boc-Leu-Lys-Arg-AMC as substrate (0.42 mM, final concentration). Calculations were carried out using the Thrombinoscope software.

generation was observed in plasmas with decreased C1-inhibitor activity (HAE plasma) compared to the EDTA pool (Fig. 4D).

4. Discussion

In this article we present a series of experiments demonstrating a possible setup for measuring FXII-dependent kallikrein generation in undiluted plasma. The results obtained by the current method are calibrated, thus the final results represent generation of kallikrein activity over time.

Michaelis-Menten kinetics of three fluorogenic substrates for kallikrein [26] were investigated to determine whether they were suitable for continuous generation experiments, *i.e.* display high K_M and low k_{cat} values [27]. As an effect of albumin on the hydrolysis of a fluorogenic substrate has been reported previously [28], the kinetic constants were determined in buffer with an albumin concentration similar to that of plasma. Under these circumstances, Boc-Leu-Lys-Arg-AMC displayed favorable kinetics for generation experiments with a high apparent K_M (0.91 mM) and a low k_{cat} (19 s^{-1}) with respect to kallikrein. Cbz-Pro-Phe-Arg-AMC and Cbz-Phe-Arg-AMC showed K_M values of 1.4 and 0.83 mM, respectively, and considerably higher k_{cat} values (45 and 33 s^{-1}, respectively). Furthermore, at low substrate concentrations, a sigmoidal curvature of initial rate versus substrate concentration was observed for both Cbz-Pro-Phe-Arg-AMC and Cbz-Phe-Arg-AMC (data not shown). These observations are likely to be due to a strong interaction between albumin and the substrates as, in the absence of albumin, the relationship between initial rate and substrate concentration was hyperbolic (Michaelis-Menten) for Cbz-Pro-Phe-Arg-AMC and Cbz-Phe-Arg-AMC (data not shown). Because albumin is abundant in plasma, the use of Cbz-Pro-Phe-Arg-AMC or Cbz-Phe-Arg-AMC may be accompanied by significant complications in the current setup.

The selectivity of Boc-Leu-Lys-Arg-AMC for plasma kallikrein compared to other serine proteases is of major importance. The selectivity of the substrate was investigated by comparing the kinetic constants for kallikrein, FXIIa, FXIa, thrombin, and plasmin (Table 2). The substrate demonstrated higher K_M for FXIa and plasmin than for kallikrein but comparable or lower K_M for FXIIa and thrombin. However, very low

k_{cat}/K_M values were observed for both FXIIa and thrombin compared to kallikrein, and a significant contribution from these enzymes upon signal generation is therefore unlikely. In addition, many of the proteases are present in plasma in much lower concentrations than that of prekallikrein. For example, the plasma concentration of FXI is around twenty times lower than that of prekallikrein [16]. Even though a two-fold difference between the k_{cat}/K_M for kallikrein and FXIa was recorded, the signal development from cleavage by FXIa will be minimal [21]. Furthermore, the initial phase of contact activation does not require presence of calcium. Consequently, re-calcification is omitted securing no or very little activation of calcium dependent proteases.

The enzymatic capacity of the α_2M-thrombin complex, used as calibrator in the CAT-method [18], is not affected by inhibitors in plasma but retains the ability to cleave small substrates. α_2M-kallikrein was likewise unaffected by inhibitors in plasma as no decrease in activity was observed after incubation in plasma for 1 h. It is preferable that the affinity of the substrate for kallikrein and α_2M-kallikrein is similar for calibration. However, complex formation between kallikrein and α_2M affected the kinetic constant K_M, which appeared lower towards the substrate when kallikrein was bound to α_2M. It is peculiar that interaction between the enzyme and an inhibitor result in an apparently improved binding affinity (*i.e.* lower K_M) of the substrate. However, this finding is in accordance with previous reports, in which a similar trend was observed for human α_2M-kallikrein [29], α_2M-trypsin [30], and α_2M-thrombin [31] complexes. The effect of difference in K_M upon calibration was not investigated here.

Polyphosphates [32] and misfolded proteins [33] have been proposed as physiological activators of the contact system, but the subject is still under study. Other assays relying on contact activation, such as the APTT and contact dependent thrombin generation, employ non-physiological activators. In light of this, we investigated several of the known non-physiological activators in the current setup. When the APTT reagent was used as activator, a moderate signal was observed. The signal diminished dose-dependently with lower concentration of APTT reagent. At high concentrations, both dextran sulfate and colloidal silica generated a stronger signal than the APTT reagent. The

use of dextran sulfate, however, resulted in significant turbidity, which presumably is caused by dextran sulfate induced precipitation of proteins. This was not observed when silica was used as the activator.

Variation in pre-analytical handling of samples can affect test consistency [34,35]. While the effects of pre-analytical factors were not investigated in the present study, we did not record any signal development in the absence of contact activator. These findings indicate that the proposed method is not affected by the surface of the microtiter plate. Furthermore, the findings also indicate that the reported activity of PK-HMWK complexes [36] is not detected by the current method.

The current analytical setup does, however, suffer from other obstacles seen in the CAT-method including effects of substrate depletion, the inner-filter effect, and cleavage of substrate by the formation of α_2M-enzyme complexes. In the CAT-method, mathematical algorithms are used to circumvent these obstacles and such algorithms should be applicable in the current setup as well. The manual calculations presented here showed that the algorithms could be used to overcome these complications.

The validation experiments strongly indicated that the observed signal was related to FXII-driven kallikrein generation. We observed no signal in plasmas depleted of FXII or PK confirming that the presence of these proteins is required. Furthermore, adding citrate pool to the FXII DEP dose-dependently restored signal generation. This indicates a considerable effect from plasma levels of FXII. The amount of C1-inhibitor activity in plasma also affected the signal generated substantially. Addition of C1-inhibitor to a citrate pool diminished signal generation, whereas plasmas with decreased C1-inhibitor activity (HAE plasma) generated a much stronger signal. These findings, in combination with the selectivity studies of the substrate, strongly suggest that the proposed method predominantly measures kallikrein activity over time.

In the present series of experiments, we have demonstrated that calibrated kallikrein generation can be determined in undiluted human plasma samples. Our experiments could lay down the basis of a functional, sensitive, and accurate global assay for measurements of the initial activation phase of the contact system. Furthermore, the experiments show that such a method could be useful to study abnormalities in the contact activation system as the signal was considerably affected by levels of FXII, PK, and C1-inhibitor.

Acknowledgments

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